

Claims

1. A method for screening a library of protein variants for functional variants with reduced antibody binding capacity, comprising the steps of:

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(i) generating a diversified library of protein variants starting from a relevant protein backbone,

(ii) transforming the library into suitable host cells,

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(iii) culturing host cells,

(iv) sampling each cell culture ,

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(v) analysing a sample by determining the antibody binding capacity of the variant protein,

(vi) analysing a sample by determining the functionality of the variant protein.

2. The method according to claim 1, wherein the following steps are added between step (ii) and (iii):

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(iib) culturing host cells,

(iic) assaying function,

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(iid) selecting host cells expressing functional protein variants.

3. The method according to claim 2, wherein the selected cells in step (iid) are picked by a colony-picker.

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- claim 1*
4. The method according to ~~claims 1-3~~, wherein the library diversity is located in epitope areas.
- claim 1*
5. The method according to ~~claims 1-4~~, wherein the protein variants are modified by substitution, addition, and/or deletion of amino acid residues suitable for chemical modification of the protein.
- claim 1*
6. The method according to ~~claims 1-2~~, wherein the protein variants are modified by introduction of one or more additional post-translational modification site and expressed in a host suitable for the corresponding *in vivo* post-translational modification.
7. The method according to claim 6, wherein the site is a N-glycosylation site or a phosphorylation site.
- claim 1*
8. The method according to ~~claims 1-7~~, where the diversified library is randomized at one or more individual positions (DNA codons) at the primer level.
9. The method according to claim 8, wherein the library is biased towards amino acids that can be chemically modified.
10. The method according to claim 8, wherein the library is biased towards amino acids that correspond to post-translational modification recognition sequences.
11. The method according to claim 10, wherein the amino acids correspond to N-glycosylation sites or phosphorylation sites.
12. The method according to claim 8, wherein the library is biased towards sequences that are not predicted to result in formation of new epitopes.
- claim 1*
13. The method according to ~~claims 1-7~~, where the diversified library is randomized by combination of segments of known sequence,

9 14. The method according to ^{claim} ~~claims~~ 1-13, wherein the library is diversified simultaneously at several discrete sites on the three dimensional structure.

5 15. The method according to claim 14, wherein the library is assayed with specific polyclonal antibodies.

16. The method according to claim 14. Wherein the library is assayed for antigen binding in an assay that requires bivalent antigen-antibody interactions.

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9 17. The method according to ^{claim 1} ~~claims~~ 1-13, wherein the library is diversified at a single site on the three-dimensional structure.

15 18. The method according to claim 17, wherein the library is assayed with a monospecific antibody.

19. The method according to claim 17, wherein the library is assayed with a monoclonal antibody.

20 20. The method according to claims 19, wherein the library is diversified at a single epitope area and assayed with a monospecific antibody purified using the corresponding peptide-phage membrane protein fusion.

21. The method according to claims 1-20, wherein the cells in step (ii) are dispensed in a
25 multi-compartment device in a dilution such that each compartment contains an average of 0,2-1,0 cells.

22. The method according to claims 1-21, wherein the sample of step (iv) is separated from the host cells by a membrane process.

23. The method according to claims 1-22, wherein the sample is analysed by determining the total content of protein variant.

24. The method according to claims 1-22, wherein the sample is analysed by exposure to
5 adverse conditions prior to determining the functionality.

25. The method according to claims 1-22, wherein the sample functionality is analysed both prior to and after exposure to adverse conditions.

10 26. The method according to claim 1-13 and 21-25, wherein the antibodies are derived from animals sensitized with the backbone protein of step (i).

27. The method according to claims 26, wherein the antibodies are derived from animals sensitized by intratracheal exposure

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28. The method according to claim 1-13 and 21-25, wherein the antibodies are derived from human volunteers that are sensitized to the backbone protein of step (i).

29. The method according to claims 26-28, wherein the antibodies are raised against the same
20 protein, but expressed in a strain different from the host cells, such as to minimize background binding to host cell impurities.

30. The method according to claims 26-29, wherein the antibodies are contained in serum from the animal or human.

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31. The method according to claim 30, wherein the antibodies are IgG, IgM and/or IgE antibodies.

32. The method according to claims 30-31, wherein the antibodies are antigen-specific
30 antibodies.

33 The method according to claim 32, wherein the antibodies are selected for the binding affinity to specific epitopes.

34. The method according to claims 30-33, wherein the antibodies are purified by capturing
5 those that bind to impurities of the culture supernatant.

35. The method according to claims 26-29, wherein the antibodies are monoclonal antibodies.

36. The method according to claim 35, wherein the clones are selected for the binding affinity
10 of their corresponding antibodies to specific epitopes.

37. The method according to claims 1-36, wherein the antibody binding is determined from a single dilution of the protein variant.

15 38. The method according to claims 1-37, wherein the functionality to be determined is enzyme activity.

39. The method according to claims 1-38, wherein the protein variants are bound to a solid phase.
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40. The method according to claim 39, wherein the solid phase is a dipstick.

41. The method according to claim 40, wherein the immobilised protein variants are transferred from one test solution to another by sequentially immersing the dipstick in the test
25 solutions.

42. The method according to claim 41, wherein the test solution(s) is(are) placed in wells, e.g. in 96 well plates.

30 43. The method according to claim 39, wherein the solid surface is a microtiter well surface.

44. The method according to claim 39, wherein the solid surface is the surface of beads.
45. The method according to claims 39-44, wherein the binding capacity of the solid surface is less than the average protein variant content of the sample such that the surface binding
5 samples a reproducible amount of protein variant for analysis.
46. The method according to claims 39-45, wherein the protein variant is linked to a fusion peptide which mediates binding to the solid phase.
- 10 47. The method according to claims 39-46, wherein the protein variant is modified by chemical conjugation prior to steps (v) and (vi).
48. The method according to claim 47, wherein the protein variant is conjugated with activated PEG.
- 15 49. The method according to claim 48, wherein the protein is conjugated to activated PEG molecules of molecular weight ranging from 100 to 5000 Da at a ratio of activated polymer to lysines in protein that is greater than 5.
- 20 50. The method according to claims 39-49, wherein the test solution of step (v) comprises antibodies and competitive backbone protein.
51. The method according to claims 39-50, wherein bound antigen is detected using a primary antigen-specific antibody and a labelled secondary antibody specific for the primary antibody.
- 25 52. The method according to claims 39-50, wherein the bound antigen is detected using a labelled primary antibody.
53. The method according to claims 50, wherein the competitor is added in an amounts equal
30 to the amount of immobilised protein variant, better at amounts that are 10x higher than the

amount of immobilised protein variant, but preferentially more than 100x higher than the amount of immobilised protein variant.

54. The method according to claims 50 or 53, wherein the selected variants show reduced
5 antibody binding in the presence of competitor, at least 5% reduced, preferably more than 10% reduced, more preferred above 50% reduced and most preferably more than 75% reduced.

55 The method according to claims 39-54, wherein the protein variant is eluted from the solid
10 phase prior to determining the functionality in step (vi).

56. The method according to claims 1-38, wherein the antibody binding is determined by agglutination of beads or cells coated with antibodies.

15 57. The method according to claims 1-38, wherein the antibody binding is determined by IgE antibodies bound to the surface of effector cells

58. The method according to claim 39-54, wherein the protein variants are bound reversibly to a solid phase and are eluted prior to analysis in (v) and (vi).
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59. The method according to claims 1-38 or claim 58, wherein antibodies are coated on a solid surface and incubated with sample.

60. The method according to claim 59, wherein labelled competitive protein is incubated
25 together with sample.

61. The method according to claim 60, wherein the amount of competitor is smaller than the average amount of protein variant in the sample.

30 62. The method according to claims 60-61, wherein the competitor is labelled chemically or genetically.

63 The method according to claims 60-62, wherein bound labelled competitor is determined after removal of the sample.

5 64. The method according to claims 1-38 or claim 58, wherein antibody binding is used to capture protein variant and the functionality is determined for the non-captured fraction.

65. The method according to claim 64, wherein binding is performed at conditions where antigen-antibody affinity is lowered, such that a moderate change in affinity may lead to non-
10 capture.

66. The method according to claims 64-65, wherein binding is performed in the presence of inactivated competitor, such that a small change in affinity may lead to non-capture.

15 67. The method according to claim 66, wherein competitor is inactivated chemically, such that it will not affect the functionality assay.

68. The method according to claim 66, wherein the competitor is inactivated by protein engineering, such that it will not affect the functionality assay.

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